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Characterization of flowering time and SSR marker analysis of spring and winter type *Brassica napus* L. germplasm

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Abstract Flowering dates and life forms of all available *Brassica napus* accessions conserved at the North Central Regional Plant Introduction Station (NCRPIS) were characterized, and a survey of molecular variation was conducted by using simple sequence repeats (SSR) in order to support better management of accessions with diverse life forms. To characterize flowering phenology, 598 *B. napus* accessions from the NCRPIS collection were planted in Iowa and Kansas field sites together with a current commercial cultivar and observed for days to flowering (first, 50% and 100% flowering) in 2003. Days from planting to 50% flowering ranged from 34 to 83 in Iowa and from 53 to 89 in Kansas. The mean accumulated growing degree days (GDD) to 50% flowering were 1,997 in Iowa, and 2,106 in Kansas. Between locations, the correlation in flowering time ($r = 0.42$) and the correlation in computed GDD ($r = 0.40$) were both significant. Differences in flowering-time rank were observed for several accessions. Accessions that failed to flower in Iowa in a single growing season comprised 28.5% of the accessions; of the flowering accessions, 100% plant flowering was not always achieved. Accessions were grouped according to flowering time. A stratified sample of 50 accessions was selected from these groups, including 10 non-flowering and 40 flowering accessions of diverse geographic origins and phenological variation. The flowering time observed in the sampled accessions when grown in the greenhouse were found to be significantly correlated to the flowering time observed in the field locations in Iowa ($r = 0.79$) and Kansas ($r = 0.49$). Thirty SSR markers, selected across 18 *Brassica* linkage groups from BrassicaDB, and 3 derived from *Brassica* expressed sequence tags (ESTs) were scored in the stratified sample. An average of three bands per SSR primer pair was observed.

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Associations of SSR marker fragments with the life forms were determined. Analysis of molecular variation by using cluster analysis and ordination resulted in recognizable, distinct groups of annual and biennial life-form types, which may have direct applications for planning and management of future seed regenerations.

Keywords *Brassica napus* · Diversity · Genebank · Microsatellites · Phenology · Rapeseed

Introduction

Brassica napus is an amphidiploid (AACC genome, $2n = 38$) that is believed to have arisen from interspecific hybridization between the diploid species *Brassica rapa* L. (syn. *Brassica campestris*; AA genome, $2n = 20$) and *Brassica oleracea* L. (CC genome, $2n = 19$) (Sauer 1993; Gómez-Campo 1999). *B. napus* has a short evolutionary history since it is presumed to have arisen from cultivation, and no true wild forms have been found (Lackey 1996; Gómez-Campo and Prakash 1999). This species is believed to have originated in the Mediterranean region of southwestern Europe where native populations of *B. rapa* and *B. oleracea* overlap. *B. napus* populations can be classified into annual or biennial forms. The annual forms are called spring rapes and the biennial forms are called winter rapes (McNaughton 1995). Spring rapes are sown and harvested in the same season, while winter rapes are sown in the fall and require vernalization before flowering in the next season (Butruille et al. 1999). It is believed that annual and winter type *B. napus* are distinct groups; intercrossing between these types is still not common even in contemporary breeding programs (Diers and Osborn 1994).

Extensive genetic resource collections of *Brassica* exist to support both public and private breeding programs (Boukema and van Hintum 1999). The USDA-ARS North Central Plant Introduction (NCRPIS) in Ames, Iowa has been mandated in the U.S. to curate germplasm of *B. napus* along with more than 20 other species of *Brassica* and their wild and weedy relatives. Both annual and biennial populations are represented among the accessions conserved by the NCRPIS, and possibly some of mixed life forms. However, not every accession is linked to passport or characterization data describing the type of life form. Information on an accession's life form prior to regeneration facilitates more efficient resource management by identifying those that require vernalization, a time and labor-intensive process that requires specialized facilities. If accessions consist of mixed life forms, regeneration methods can be modified to preserve their genetic profiles. To date, all types have been subjected to vernalization treatment and then transplanted to the field as part of standard regeneration procedures. More accurate information on the flowering data of annual types can be collected without vernalization, since exposure to cold treatment may lead to precocious flowering (Friend 1985; Sovero 1993). At present, the available *B. napus* flowering data on the ARS-GRIN database (http://www.ars-grin.gov/npgs) indicates 'days to flower' which include the number of days from the time accessions are germinated in blotter boxes, vernalized, and transplanted to the field. To complement the existing data in the GRIN database, we conducted this study and gathered flowering data on annual accessions without vernalization. The specific objectives are to (i) determine life forms of all available *B. napus* accessions by using morphological and phenological characterization, (ii) survey the molecular genetic variation of representative accessions, and (iii) identify life-form specific markers to possibly help curators in screening new accessions quickly without waiting for actual flowering.

To address the last two of the aforementioned objectives, simple sequence repeats (SSR) or microsatellite markers were used. SSRs are fairly robust, have good reproducibility, and are more cost-effective compared to other marker systems (Farooq and Azam 2002). It is anticipated that the GRIN database will include SSR profiles of germplasm accessions; some crops already have SSR data. In *Brassica*, SSRs have been valuable tools, and numerous SSR primer sequences are already publicly available (Snowdon and Friedt 2004). SSRs have been used in *Brassica* research

for such topics as seed-coat color mapping (Padmaja et al. 2005), varietal identification (Tonguç and Griffiths 2004), and analysis of variation in plant populations and germplasm collections (Raybould et al. 1999; Westman and Kresovich 1999). It was also reported that a set of SSRs were able to identify groups of annual and biennial types of *B. napus* elite lines (Plieske and Struss 2001; Tommasini et al. 2003). Here we test whether it is also possible to identify life-form types using diverse, heterogeneous germplasm collections of *B. napus*.

Methodology

Planting and characterization of flowering time

In May 2003, 598 accessions of *B. napus* originating from 28 countries were planted at the NCRPIS farm in Ames, Iowa together with 'Hyola 401', a commercial spring cultivar. In Ames, the accessions were randomized and planted in three replicates by direct seeding in 7-m, single-row plots in a 0.78 ha field located at 42°00′29.61″N, 93°39′48.82″W. In April 2003, these same 598 accessions were also planted in 7-m, single-row plots without replication (due to resource limitations) at Kansas State University (KSU), Manhattan, Kansas. Data gathered in Iowa included: (1) date of 50% germination, (2) date of first flowering (when the first plant in the plot flowered), (3) date of 50% flowering (when 50% of the plants in the plot flowered), and (4) date of 100% flowering. Data gathered in Kansas included date of 50% flowering. Following characterization during the 2003 field season, 50 accessions representing the range of flowering times observed as well as diverse geographic sources were selected (Table 1). These accessions were comprised of 10 accessions of non-flowering types (putative biennials) and 40 of flowering types (putative annuals). Selection was done by hierarchical clustering of the flowering time and subsequent selection from each of the resulting eight clusters based on geographic origin. The selected accessions included cultivars and breeding lines. All of the selected representative accessions from among the flowering types attained at least 50% flowering in the field in both locations.

In March 2004, seeds from the original seed lot of the selected accessions were sown in 10 cm^2 plastic pots in the greenhouse containing Sunshine Mix no. 1 (Sun Gro Horticulture, Bellevue, WA). Twenty-four plants for each accession were grown under a 16-h photoperiod to verify the life form, determine correlations between flowering time in the greenhouse and the field, and to obtain leaf tissues for molecular-marker analysis. Mean greenhouse temperature and illuminance were recorded at 26°C and 3.9 klux, respectively, by HOBO® H08-004-02 data loggers (Onset Computer Corporation, Bourne, MA). The data loggers were positioned at the leaf canopy level.

Plant material and DNA extraction

Leaf tissues were harvested when the plants in the greenhouse were at the 3–4 leaf stage, when the leaves contain relatively low amounts of polysaccharides (Hyam 1998). Twelve to 15 plants were sampled per accession. The leaves were lyophilized by freeze-drying and then stored in a –80°C freezer until use. Genomic DNA extraction was performed by using 1.0 g bulked tissue obtained from equal weights of freeze-dried leaf samples from individual plants within an accession. The bulked tissues were placed in 50 ml screw-cap polypropylene tubes, each containing approximately 1.5 ml glass beads. The tissue samples were ground to a powder by agitating the tubes on a paint shaker for 90 s. DNA was isolated using the cetyltrimethyl ammonium bromide (CTAB) method. The extracted DNA was resuspended in 1× TE (1 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) with 3 μl of 10 mg/ml RNAseA then incubated at 30°C for 30 min and placed on a LabQuake® shaker (Barnstead Intl., Dubuque, IA) at 4°C overnight. The samples were stored at –20°C until use.

PCR amplification of microsatellites

Microsatellite primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) based on published primer sequences from Uzunova and Ecke (1999), Westman and Kresovich

Table 1 List of accessions selected from the *B. napus* germplasm collection at NCRPIS

Plot ID[a]	Accession	Variety	Source[b]	DAP to 50% flower[c]
13N	Ames 6100	Jupiter	Canada	–
16N	Ames 15650	Arco C10-2	Netherlands	–
19N	Ames 15654	Bienvenu	United States	–
21	Ames 15939	Comet	Sweden	48
29N	Ames 19202	Krasnodarskii	Russia	–
31	Ames 19204	Evvin	Russia	41
32	Ames 19205	Kovalevskij	Ukraine	46
36	Ames 26653	Westar	United States	38
62	PI 311727	Bronowski	Poland	53
88	PI 436555	Gan You no. 2	China	59
92N	PI 443015	Gry	Norway	–
98N	PI 458610	Wilhelmsburger	New Zealand	–
100	PI 458919	Brio	France	44
108	PI 458930	Oro	Canada	48
109N	PI 458935	Brink	Sweden	–
113	PI 458940	Chisaya natane	Japan	83
114	PI 458941	Norin 16	Japan	68
119	PI 458948	Gisora	Germany	60
126	PI 458955	Prota	Germany	41
138	PI 458971	Romeo	France	39
173	PI 469756	Colza	South Korea	41
174	PI 469757	Colza 18 Miroc	South Korea	78
175	PI 469758	Dae cho sen	South Korea	68
179	PI 469762	Dong Hae 2	South Korea	73
189	PI 469772	Dong Hae 16	South Korea	75
193	PI 469776	Dong Hae 21	South Korea	60
205	PI 469788	Fertodi	South Korea	66
214	PI 469797	France 9	France	53
239	PI 469822	Iwashiro-natane	South Korea	66
243	PI 469826	Janetzkis	South Korea	63
276	PI 469859	Kuju 25	South Korea	40
298	PI 469881	Kuju 58	South Korea	76
303N	PI 469886	Lenora	South Korea	–
311	PI 469894	Mali	South Korea	35
327	PI 469911	Mokpo 5	South Korea	65
340	PI 469924	Mokpo 21	South Korea	59
346	PI 469930	Mokpo 27	South Korea	81
349	PI 469933	Mokpo 30	South Korea	69
356	PI 469940	Murame nadame	South Korea	36
371	PI 469955	Norin #4	Japan	79
391	PI 469975	Norin 21	Japan	45
397	PI 469981	Norin 26	Japan	55
447	PI 470031	Su weon cheg	South Korea	79
457	PI 470041	Taiwan 2	Taiwan	40
489	PI 470075	7003–2B-38	South Korea	83
502	PI 478340	O 84	China	42
539N	PI 535866	Silesia	Czechoslovakia	–
573N	Ames 22547	Bolko	Poland	–
555	PI 542984	Tri-Bridger	United States	59
574	Ames 22548	Bronowski	Poland	51

[a] Field plot number, 'N' suffix denotes non-flowering

[b] Country origin in ARS-GRIN; Bienvenu and Westar's origin were changed to France and Canada, respectively (Diers and Osborn 1994) in subsequent analyses

[c] Mean DAP values

Table 2 List of the microsatellites analyzed, repeat motifs, size range of the amplified bands approximated from molecular weight markers, and the number of bands observed in the bulked samples

Microsatellite	Repeat	Size range (kb)	Total no. bands
Na10-B08	$(CT)_{38}$	100–175	5
Na10-D03	$(GT)_{11}$	150–190	2
Na10-E02	$(GA)_{24}$	125–200	3
Na12-A02	$(CT)_{16}$	150–200	3
Na12-A07	$(GT)_{11}$	150–175	2
Na12-A08	$(GA)_{28}$	150–300	4
Na12-C07	$(CT)_{33}$	200–250	3
Na12-C08	$(CT)_{50}$	275–350	3
Na12-F03	$(GA)_{35}$	300–350	2
Na14-C12	$(AG)_{17}$	190–200	3
Na14-D07	$(CCG)_{3}$	150–175	2
Ni4-D09	$(CT)_{25}$	175–200	2
Ol10-A05	$(GA)_{43}$	110–275	7
Ol10-F11	$(GGC)_{7}$	150–175	2
Ol10-F12	$(CT)_{64}$	100–225	4
Ol11-C02	$(GT)_{11}$	140	1
Ol11-H02	$(AAC)_{18}$	180–200	2
Ol12-E03	$(CCG)_{9}$	110–250	3
Ol12-G04	$(TC)_{24}$	100–175	2
Ra2-D04	$(CA)_{14}$	160–190	2
Ra2-E03	$(CT)_{18}$	225–275	2
Ra2-E07	$(GA)_{19}$	100–170	4
Ra2-F11	$(CT)_{34}$	190–300	4
Ra2-G09	$(CT)_{19}$	200–300	3
Ra3-H10	$(GA)_{23}$	100–150	3
MR176[a]	n.d.	120–290	8
MR181[a]	$(AG)_{36}$	100–190	5
35D[b]	$(GA)_{13}$	200–250	2
59A1[b]	$(CA)_{11}$	450	1
25A[b]	$(CT)_{10}$	125–350	3
EST1[c]	n.d.	90	1
EST2[c]	n.d.	280	1
EST3[c]	n.d.	90–115	4

Sequences were obtained from BBSRC BrassicaDB, except as noted; n.d.: not determined

[a] Uzunova and Ecke (1999)

[b] Westman and Kresovich (1999)

[c] A. Salywon (USDA-ARS, U.S. Water Conservation Laboratory, Phoenix, AZ)

(1999) and the BBSRC BrassicaDB (http://brassica.bbsrc.ac.uk). The 33 microsatellite loci are presented in Table 2. To ensure representation of several linkage groups, the microsatellites from BBSRC were selected based on indicated linkage map locations in BrassicaDB and from Lowe et al. (2004). These microsatellites were developed mostly from genomic libraries (Lowe et al. 2004). Three primer pairs derived from *Brassica* EST sequences were provided by Dr. Andrew Salywon (USDA-Agricultural Research Service, U.S. Water Conservation Laboratory, Phoenix, AZ) (see Salywon et al. 2004 for detailed descriptions).

PCR was performed in 96-well Microseal™ polypropylene microplates (Bio-Rad Lab Inc., Hercules, CA) with each sample well containing 1 μl of genomic DNA (50 ng/μl), 8 μl of sterile ddH2O, 1 μl 10× PCR buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl), 0.5 μl dNTPs (2 mM), 0.3 μl MgCl (50 mM), 0.1 μl of primers (50 uM), and 0.05 μl *Taq* polymerase (5 U/μl) (Invitrogen Corp., Carslbad, CA). A negative control, a sample from a commercial hybrid (Hyola 401), and samples from three rapid-cycling *Brassica* accessions (*B. oleracea* TO 1000 DH3, *B. rapa* IMB 218 DH3, *B. napus* EL 6400 A) from Dr. J. Chris Pires (Univ. Missouri,

Columbia, MO) were included in each microplate. Thermal cycling was done by using DNA Engine® (PTC-200™) thermal cyclers (Bio-Rad Lab Inc., Hercules, CA) under the following conditions: 94°C for 2 min, then followed by 35 cycles of amplification at 94°C for 30 s, 55.5°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 4 min. Different annealing temperatures were used on two of the EST-derived primers, 53.4°C for EST 1 and 59.5°C for EST 2. All reactions with no initial amplifications were repeated to confirm the results. The PCR products were separated in a 4.0% agarose gel prepared in 1× TAE (40 mM Tris–acetate, 1 mM EDTA, pH 8.3) with incorporated ethidium bromide (0.46 μg/ml). Amplification products were visualized with a UV light box (254 nm wavelength) and photographed on a digital gel-documentation system. The bands were scored as present (1) or absent (0) and recorded with reference to the molecular weight markers. They were then treated for analysis as if they were dominant markers, since it was not possible to calculate gene frequencies as a result of bulking 12–15 plants per accession.

Data analysis

Five accessions were not included in subsequent analyses after determining that they were not likely to belong to the *B. napus* species; two accessions are probable members of *B. rapa* (PI 286418, Ames 21490), two of *B. juncea* (L.) Czern (Ames 19197, Ames 24222) and one of *B. oleracea* L. (PI 357374). Likewise, changes in country of origin of two accessions (see note Table 1) were used in the computation of geographic distances. Descriptive statistics and preliminary cluster analysis of flowering data were obtained from JMP® version 5.1.2 software (SAS Institute, Cary, NC). Growing degree days (GDD) were computed for all flowering accessions by using the formula GDD = (MinT + MaxT)/2 – BaseT, where MinT is the lowest temperature of the day and MaxT is the highest temperature of the day; temperature thresholds for MinT and MaxT were set to 0°C and 30°C, respectively. BaseT is the temperature below which no development occurs and was set to 0°C, based on recent research which indicated that it was more accurate for *B. napus* plant development than is the typical base temperature of 5°C (Thomas 2003).

Because of bulking, analysis of SSR bands was done following a shared-alleles method. Nei and Li (1979) distance were computed using the formula $d_D = 1–(2v_{ij}/2v_{ij} + w_{ij} + x_{ij})$, where v_{ij} is the number of bands in common between both accessions; w_{ij} is the number of bands present in the ith accession and absent in the jth accession; x_{ij} is the number of bands absent in the ith accession and present in the jth accession (Rief et al. 2005). Cluster analysis was performed by using the neighbor-joining method algorithm in the NTSYS-pc version 2.20e software package (Rohlf 2005). A χ^2-test for independence was done to test the hypothesis that observed band frequencies were independent of life form. Associations between band presence and flowering time were made using the non-parametric Mann–Whitney-*U* test (equivalent to Wilcoxon rank sum test) (Gebhart et al. 2004). Mantel tests were performed to determine the correlation between distance matrices (Koenig 1999) and analysis of molecular variance (AMOVA) to test for genetic differentiation. The last two aforementioned tests, as well as calculations of genetic distances based on shared alleles (Maguire et al. 2002) and geographic distances (computed using latitude–longitude coordinates of the accessions' origin), and principal coordinate analysis (PCA) were done by using the GenAlEx v.6 software package (Peakall and Smouse 2006).

Results

Characterization of flowering time

One hundred sixty-nine accessions, which represent 28.5% of the *B. napus* collection, did not flower in Iowa. Four hundred twenty-five accessions (71.5%) reached first flowering, 279 accessions (47.0%) continued to 50% flowering, and 198 accessions (33.3%) achieved 100% flowering. In the Ames location, the mean number of days after planting (DAP) to 50% germination was 13 days and the mean DAP for first flowering was 49. For the 279 accessions that continued to the 50% flowering stage, the mean number of DAP

was 55. The remainder did not reach that stage by 100 DAP when scoring ended. For the 198 accessions that achieved 100% flowering, the mean number of DAP was also 55. This reflects the phenomenon that those accessions that most quickly reached the 50% flowering stage also generally achieved 100% flowering first. For those accessions that achieved 100% flowering, on average, it took 7 days from first to 50% flowering, and 3 days from 50% flowering to 100% flowering. PI 537302 was the earliest flowering accession, attaining 50% flowering at 34 DAP, while the very late flowering accessions PI458940, PI 469770, and PI 470075 attained 50% flowering at 83 DAP. The plants of about 32% of the flowering accessions flowered synchronously (Fig. 1**A**). In Kansas, 50% flowering was observed in 223 accessions with a mean of 74 DAP. In general, more *B. napus* accessions flowered late when planted in Kansas (Fig. 1). Correlation between the 50% flowering dates observed in Iowa and Kansas was low ($r = 0.42$) but significant ($p < 0.0001$). There was a mean difference of 4 days between 50% flowering in the two locations. The commercial cultivar Hyola 401 is the earliest to attain 50% flowering followed by Ames 26654 at 53 DAP and 54 DAP, respectively. Sixteen accessions reached 50% flowering at 89 DAP. The flowering times observed in the greenhouse for 50 selected representative accessions were found to be correlated to flowering times observed in the field in both Iowa ($r = 0.79$, $p < 0.0001$) and Kansas ($r = 0.43$, $p = 0.008$). However, the mean DAP to flowering in the greenhouse were 62 to first flowering and 92 to 50% flowering, indicating that temperature and photoperiod differences may have delayed the development of flowering in a spring greenhouse relative to summer field conditions.

Accumulated GDDs to flower were computed for all flowering accessions. GDDs can better describe the conditions required to reach physiological developmental stages, such as flowering, than 'days to maturity' alone (Eckert 2005). For data from Iowa, the accumulated GDDs were determined as follows: 207 ± 2 (mean ± s.e.) for 50% germination, 1008 ± 9 for first flowering, 1997 ± 29 for 50% flowering, and 2021 ± 28 for 100% flowering. For data from Kansas, the accumulated GDD for 50% flowering was 2106 ± 22. Correlation between the accumulated GDDs to 50% flowering in the two locations is low but significant ($r = 0.40$, $p < 0.0001$). A scatter-plot matrix of the GDDs in the two locations is shown in Fig. 2. The estimates of GDDs in the two locations used in this study exceed the means and range of GDDs for each growth stage compiled from research data of Agriculture and Agri-Food Canada (AAFC) for *B. napus* (Thomas 2003). The large mean values might have been influenced by the predominance of mid- and late-flowering accessions in the collection relative to those cultivated in Canada.

Microsatellite analysis

A total of 98 bands and an average of 3 bands per primer pair were observed based on all 33 SSRs and bulked samples from 50 selected accessions. The primer pairs all showed amplification, but band presence in EST1 was observed in only one accession. No polymorphism was observed in SSR 59A1. Single-band products were observed in four of the SSR primer pairs, while the remaining primer pairs amplified two to eight products (Table 2). The number and size range of observed bands amplified from the bulked tissues were consistent with other studies (Allender 2004; Tommasini et al. 2003). Across the 50 selected accessions, 71.9% of bands showed presence absence polymorphism. The rate of polymorphism was higher among the flowering types, 82.7%, than for the non-flowering types, 61.2%. A χ^2-test for independence indicated that there were significant differences in band frequencies between the non-flowering and flowering types ($p < 0.0001$). Seventeen bands from 11 SSRs were observed in only one type of life form; 12 bands were observed solely in flowering types and 5 solely in the non-flowering types. These bands were from SSRs 25A, EST3, MR176, Na10-B08, Na12-A08, Na12-C07, Na14-C12, Ol10-F11, Ol12-G04, Ra2-E07, and Ra3-H10.

The observed frequencies of the unique bands in the non-flowering types were very low. However, results of Mann–Whitney-*U* tests indicated that band presence in EST2, Na10-B08,

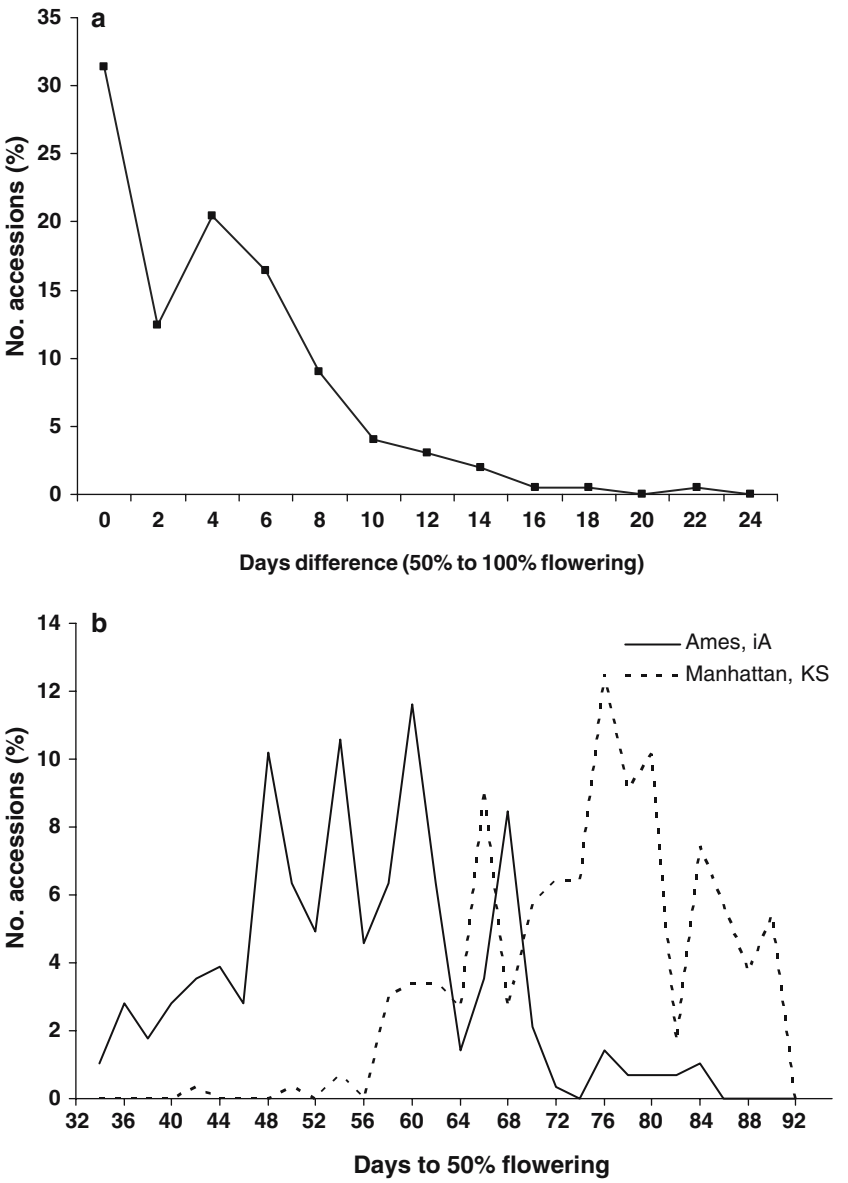

Fig. 1 Graph of flowering time showing the proportion of 224 accessions versus (**A**) the number of days between 50% and 100% flowering in IA and (**B**) the number of days after planting to 50% flowering in IA and KS

Ol11-C02, Ra2-E07, and Ra2-F11 exhibited significant associations with flowering time (Table 3).

A plot of the first two principal coordinates (PCO) derived from computed genetic distances from SSR-marker profiles allowed the visualization of relationships among accessions (Fig. 3). Axis 1 explains 25.81% of the observed variation and axis 2, 18.35%. The non-flowering types grouped in quadrant IV, as did known early flowering cultivars in quadrant I. The pattern observed in the PCO plot closely resembles relationships among accessions when cluster analysis was performed. The dendrogram separated flowering and non-flowering types, and early flowering accessions mostly grouped together (Fig. 4). Among the non-flowering types, only 539N (PI 535866, Silesia) was not within the same cluster. No distinct clustering of mid- or late-flowering accessions was observed. The computed, mean distance among the non-flowering types was 0.23, and among the flowering types it was 0.28. The smallest distance observed was 0.06 between 31 (Ames 19204) and 32 (Ames 19205); both originated from the former Soviet

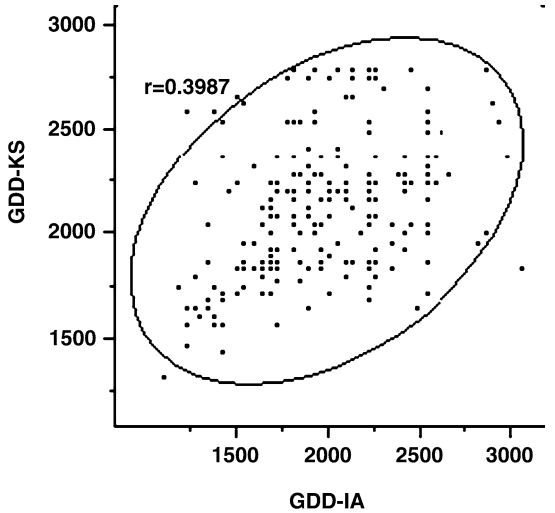

Fig. 2 Scatter-plot matrix of accumulated growing degree days (GDDs) to 50% flowering in Iowa and Kansas locations (density ellipse shown with $\alpha = 0.95$)

Union. Other accessions with the same country of origin also grouped together: 113 (PI 458940) and 114 (PI 458941) from Japan, 119 (PI 458948) and 126 (PI 458955) from Germany, and 62 (PI 311727) and 574 (Ames 22548) from Poland. Accessions with the same variety name also grouped together: 62 (PI 311727) and 574 (Ames 22548)—'Bronowski', and 327 (PI 469911), 340 (PI 469924), and 349 (PI 469933)—'Mokpo'.

The dendrogram indicates greater similarity between the rapid-cycling *B. oleracea* accession and representatives of *B. napus* as compared to the *B. rapa* control. This observation supports a phylogeny proposed by Pradhan et al. (1992) among the *Brassica* species that was derived from analyses of cpDNA and mtDNA polymorphisms. 'Hyola 401' is in a cluster with the early flowering accessions and known commercial canola cultivars, including 36 (Ames 2665, 'Westar'), 21 (Ames 15939, 'Comet'), and 108 (PI 458930, 'Oro'). Pedigree information was not available in the passport data of the canola cultivars, but their clustering may be due to highly similar genetic background. It has been suggested that there is a single common origin for most of the oilseed *B. napus* cultivars from results of RFLP analysis (Song and Osborn 1992).

Results of Mantel tests indicated that the computed genetic distances is weakly correlated with geographic distances between the countries of origin ($r = 0.35, p < 0.001$). The significance of these correlations indicates that accessions with proximate geographic origin are somewhat more likely to have similar genetic profiles. Results of analysis of molecular variance (AMOVA) among different levels are presented in Table 4. The population genetic differentiation based on the binary data was given as Φpt, which is analogous to *Fst* (Peakall and Smouse 2006). *Fst* values range from 0, when the subpopulations are identical in allele frequencies to 0.5 if they are fixed for different alleles. The computed values considering life forms (Φpt = 0.11) and geographical origin (Φpt = 0.02–0.12) indicate that there is a low to moderate genetic differentiation between the specified groupings. Grouping by life forms and by geographical regions explain 11% and 6% of the molecular variation, respectively. Much of the differentiation remained within the groupings in all comparisons with explained variation ranging from 88% to 98%. The results of the AMOVA analysis imply that there is some gene flow between life forms and more extensive gene exchange among geographic regions. Diers and

Table 3 Results of Mann–Whitney-*U* tests for association

Non-specific and specific bands in five SSRs with significant association to flowering time

SSR	Band no.	Size (kb)	Band presence (%)		Probability
			Non-flowering	Flowering	
EST2	1	280	70	47.5	0.01
Na10-B08	1	100	30	42.5	0.00
Ol11-C02	1	140	60	85	0.04
Ra2-E07	2	150	–	20	0.03
Ra2-E07	4	100	–	37.5	0.04
Ra2-F11	1	190	30	35	0.01

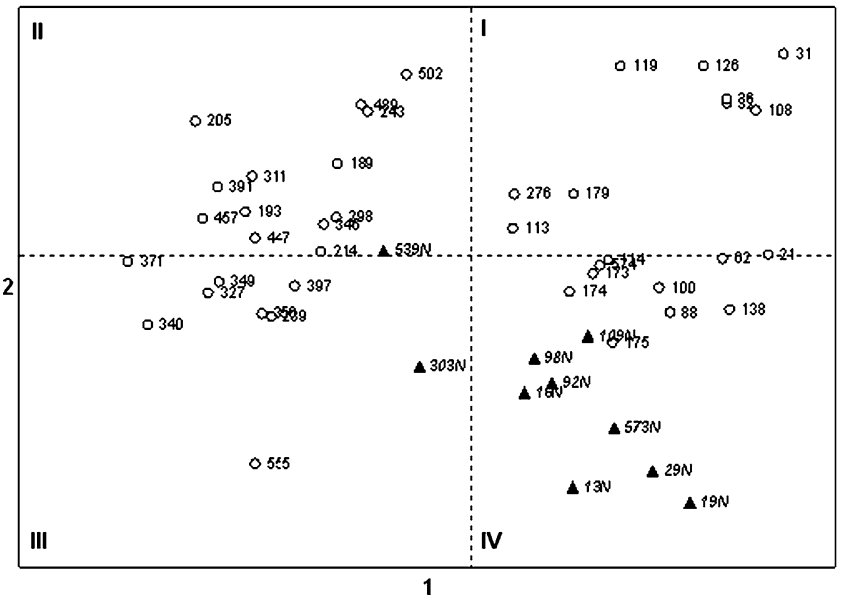

Fig. 3 Plot of variation in flowering (○) and non-flowering (▲) accessions using eigenvectors derived from genetic distances by using shared alleles. Labels correspond to identification listed in Table 1

Osborn (1994) indicated that intercrossing between annual and winter type *B. napus* is still not common even in contemporary breeding programs. The data obtained from clustering and principal coordinate analyses as discussed above support Diers and Osborn's statements that these *B. napus* life forms are distinct groups.

Discussion

In the conservation of plant genetic resources, the availability of characterization data and information on available diversity help germplasm users identify the accessions of interest and also provide plant breeders initial data for use in crop improvement programs. In this study, a survey of molecular variation was conducted and additional information was obtained to determine the life form of the *B. napus* accessions in the collection. The observed differences in the flowering time of annual accessions in the two locations gave some information on the effect of environment on the flowering of *B. napus*. The flowering time differences might have been caused by the varying responses of the genotypes to temperature and photoperiod (Friend 1985). Such differences suggest that it is desirable to conduct characterization and evaluation trials for *Brassica* germplasm in more than one location, with distinct temperature and photoperiod regimens if resources permit. In the model species *Arabidopsis*, photoperiod and temperature affect the variation in flowering time by targeting expression of the *CONSTANS* and *FLOWERING LOCUS C* (*FLC*) genes located in different pathways. The major gene in the *Arabidopsis* photoperiod pathway is the *CONSTANS* gene, while in the autonomous and vernalization pathways the major gene is *FLC* (Simpson et al. 1999). Research progress in *Arabidopsis* has enabled the identification and cloning of related genes in *Brassica* that are known to directly influence the onset of flowering (Lagercrantz et al. 2002; Martynov and Khavkin 2004, Osborn and Lukens 2003). In *Brassica*, *CONSTANS* has been proposed to have the greatest influence in the flowering time variation in *B. nigra* and *B. oleracea* (Bouhon et al. 1998; Lagercrantz et al. 1996). In *B. napus*, studies suggest that it is the *FLC* gene that influences flowering time in this species (Osborn and Lukens 2003). Exposure to vernalization treatment diminishes the effect of late-flowering alleles of *FLC* (Osborn and Lukens 2003). We have also examined and compared the *FLC* sequence variation between spring and winter types using the same set of accessions in this study, the results will be published in another paper.

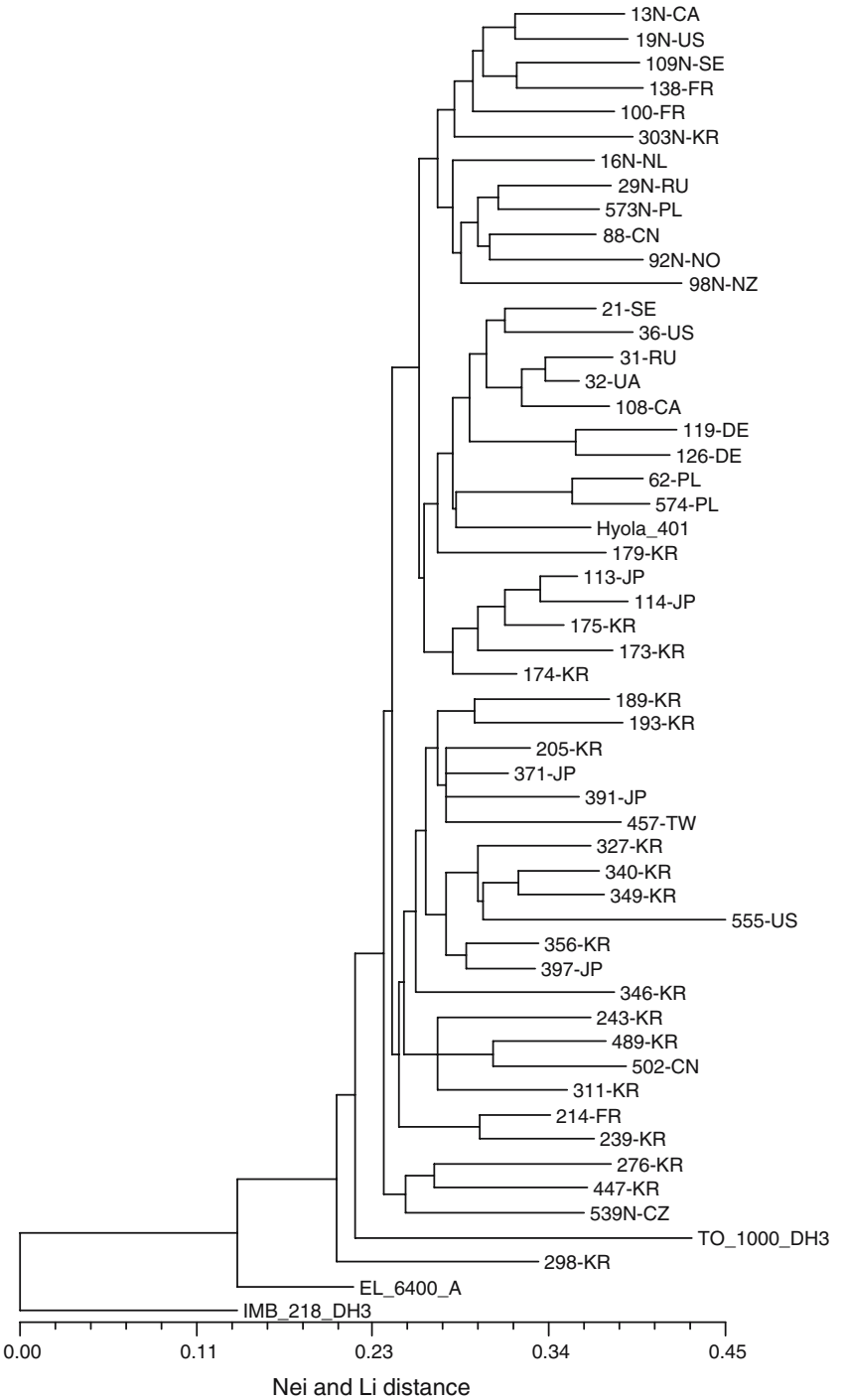

Fig. 4 Neighbor-joining tree showing the relationships among the selected *B. napus* accessions and rapid-cycling lines (*B. oleracea* TO 1000 DH3, *B. rapa* IMB 218 DH3, *B. napus* EL 6400 A). Labels correspond to plot numbers listed in Table 1 with added suffixes indicating country origin (in two letter ISO codes)

In several accessions of *B. napus* germplasm that we have regenerated in this study, the observed differences in flowering time within accessions can be a concern for germplasm managers. The heterogeneity of flowering within accessions during regeneration has the potential to influence changes in allelic frequency and genetic profile over time, through assortative mating and possible selection against infrequent phenological phenotypes or those that do not

Table 4 AMOVA by life form (non-flowering versus flowering) and by geographic regions (Europe, North America and Asia-Pacific-Oceania)

Source	df	SS	MS	Est. var.	%	Φpt	Probability
Life forms							
Among life forms	1	33.320	33.320	1.390	11	0.111395	0.001
Within life forms	48	532.100	11.085	11.085	89		
Geographical regions							
All accessions							
Among regions	2	40.207	20.103	0.677	6	0.057157	0.002
Within regions	47	525.213	11.175	11.175	94		
Flowering accessions only							
Among regions	2	46.755	23.378	1.498	12	0.124442	0.001
Within regions	37	389.845	10.536	10.536	88		
Non-flowering accessions only							
Among regions	2	22.000	11.000	0.179	2	0.016722	0.399
Within regions	7	73.500	10.500	10.500	98		

flower under local conditions. Significant shifts in phenotype frequencies have been documented to occur in *B. napus* with just one cycle of germplasm regeneration (Diaz et al. 1997). In the present study, more than half of all flowering accessions did not progress to complete flowering. Only plants that flowered will be represented in the regenerated sample, resulting in possible genetic shifts. Non-flowering of spring types and of vernalized winter *Brassica* has also been documented and attributed to the effects of high temperatures which often cause devernalization (Dahanayake and Galwey 1998). If needed, the floral induction can be accomplished through the application of gibberellins at the *B. napus* rosette stage (Dahanayake and Galwey 1999).

We have determined that it is possible to classify diverse accessions of *B. napus* into life-form types using SSRs. This finding corroborates past results of studies in breeding lines and elite cultivars of winter and spring types (Charters et al. 1996; Lombard et al. 1999; Plieske and Struss 2001; Tommasini et al. 2003). Our study further demonstrates that a limited set of SSRs can generate sufficient variability to distinguish among the life-form types in our set of germplasm. The data generated using SSRs may also prove useful in breeding programs. The results of the SSR analysis may have applications in screening new sets of *B. napus* accessions without life form data or possibly in identifying early or late flowering accession. The usefulness of marker–trait associations in assessing the genetic potential in germplasm collections has been previously studied in crops such as alfalfa (Skinner et al. 2000), cotton (Abdurakhmonov et al. 2005), potato (Gebhart et al. 2004), and rice (Kadirvel and Gunathilagaraj 2003). In *B. napus*, it has been demonstrated that molecular markers can be used in identifying genotypes with certain linolenic and erucic acid levels (Rajcan et al. 1999) and in predicting hybrid performance as suggested by significant correlations between agronomic traits, such as plant height and seed yield, and genetic distances from markers (Riaz et al. 2001; Yu et al. 2005).

Summary and conclusions

Characterization of flowering time in the field enabled the identification of life forms of genebank accessions of *B. napus* that had not been previously regenerated. The flowering dates obtained will be made available to complement existing data on the ARS-GRIN database. Our flowering dates should better represent a true phenology for the annual types, since the seedlings were not vernalized. An observed shift in flowering time in several accessions when planted in different locations was observed; this indicates that future flowering data characterizations

should be conducted by using multiple sites and interpreted accordingly.

Clustering based on marker profiles derived from microsatellites segregated groups of life forms and also identified probable duplicates (accessions with the same name). Results of the ordination analysis and AMOVA revealed that diversity 'within' life forms is greater than 'between' types of life forms in our selected set of representative accessions, which is in agreement with results from the clustering analysis. Significant, but relatively weak associations between derived genetic distances and geographic origins and between derived genetic distances and life forms were found. Seventeen bands from 12 SSR loci were observed to be uniquely present within accessions exhibiting a particular life form, but often these occurred with low frequencies. Additional evaluation of 11 SSR loci identified significant associations of alleles with flowering time. Additional investigation is needed to determine if these are in non coding regions, proximal to or within genomic regions that control vernalization and flowering response, or in linkage disequilibrium with such regions.

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